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Circadian clocks, clock networks, arylalkylamine *N*-acetyltransferase, and melatonin in the retina

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Abstract

Circadian clocks are self-sustaining genetically based molecular machines that impose ~24 h rhythmicity on physiology and behavior that synchronize these functions with the solar day–night cycle. Circadian clocks in the vertebrate retina optimize retinal function by driving rhythms in gene expression, photoreceptor outer segment membrane turnover, and visual sensitivity. This review focuses on recent progress in understanding how clocks and light control arylalkylamine *N*-acetyltransferase (AANAT), which is thought to drive the daily rhythm in melatonin production in those retinas that synthesize the neurohormone; AANAT is also thought to detoxify arylalkylamines through *N*-acetylation. The review will cover evidence that cAMP is a major output of the circadian clock in photoreceptor cells; and recent advances indicating that clocks and clock networks occur in multiple cell types of the retina.

Contents

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1. Introduction

Circadian rhythms are changes in biological processes that occur on a daily basis; they are driven by autonomous circadian clocks. These rhythms provide a selective advantage to organisms by allowing them to anticipate temporal changes in their environment. Circadian clocks are self-sustaining molecular/cellular oscillators that are entrained to the solar night–day cycle by environmental cues. The daily light/dark cycle is generally considered to be the most important and powerful entrainment cue, referred to as a zeitgeber; in addition, other stimuli, such as temperature cycles, feeding schedules, and social interactions can affect clock timing. Although circadian clocks can be reset by zeitgebers, they continue to cycle in constant darkness or constant lighting with a period of ~24 h. The downstream effects of these clocks on physiological processes can be 'masked' by light, which can block transmission of signals from the clock to clock-driven functions. Circadian clocks affect most aspects of vertebrate physiology and behavior by generating daily cycles in sleep and alertness, body temperature, hormone secretion, metabolism, blood pressure, intraocular pressure, and visual sensitivity (Hastings et al., 2003). In mammals, most of these rhythms are synchronized by a "master clock" in the suprachiasmatic nucleus (SCN) of the hypothalamus (Klein et al., 1991); in birds, the SCN acts in concert with other circadian clocks to control daily schedules in function (Cassone and Menaker, 1984).

1.1. Molecular basis of the circadian clock

The current model for the molecular basis of the circadian clock in all biological systems consists of transcriptional–translational feedback loops involving a highly conserved set of "clock genes". In the mammalian SCN, two such interlocking transcription–translation feedback loops regulate the firing rate of SCN neurons and surrounding hypothalamic relay nuclei (Fig. 1; for recent reviews see Reppert and Weaver, 2002; Roenneberg and Merrow, 2003; Honma and Honma, 2003; Hastings et al., 2003; Emery and Reppert, 2004). These feedback loops consist of positive and negative components. The positive components include the basic helix-loop-helix (bHLH)-PAS domain transcription factors, CLOCK and BMAL1. These transcription factors heterodimerize, bind to circadian E-box promoter elements containing the core nucleotide sequence CACGTG, and enhance the transcription of genes encoding the negative components PERIOD 1,2 and CRYPTOCHROME 1,2. The CRYPTOCHROME (CRY) and PERIOD (PER) proteins feedback inhibit the transcription of the *Cry* and *Per* genes by blocking CLOCK/BMAL1-mediated transactivation. The temporal delay between transcription and translation of the negative components, as well as some poorly understood phosphorylation events controlling nuclear import/export and protein degradation, results in a daily rhythm in the transcripts and protein products of the *Per* and *Cry* genes.

The second feedback loop involves the transactivation of the *Rev − erbα* and *Rora* genes by CLOCK/BMAL1. The protein products of these genes compete for binding to Rev-erb/Ror elements (RREs) in the *Bmal1* promoter, driving a daily rhythm of *Bmal1* transcription and closing the second feedback loop. Rhythmic expression of these clock gene products produces circadian clock outputs by regulating transcription of clock-controlled genes (CCGs). At least some of these CCGs contain circadian E-boxes and are activated rhythmically by CLOCK/BMAL1 (reviewed by Munoz and Baler, 2003).

1.2. Peripheral clocks

Many peripheral tissues rhythmically express clock genes and have functional "peripheral clocks" (Yamazaki et al., 2000), which contribute to many aspects of circadian physiology and may be synchronized by the master clock in the SCN (Hiroshige et al., 1991; Terman et al., 1993; Mistlberger, 1994; Sakamoto et al., 2000). The SCN is not obligatory for circadian rhythms in

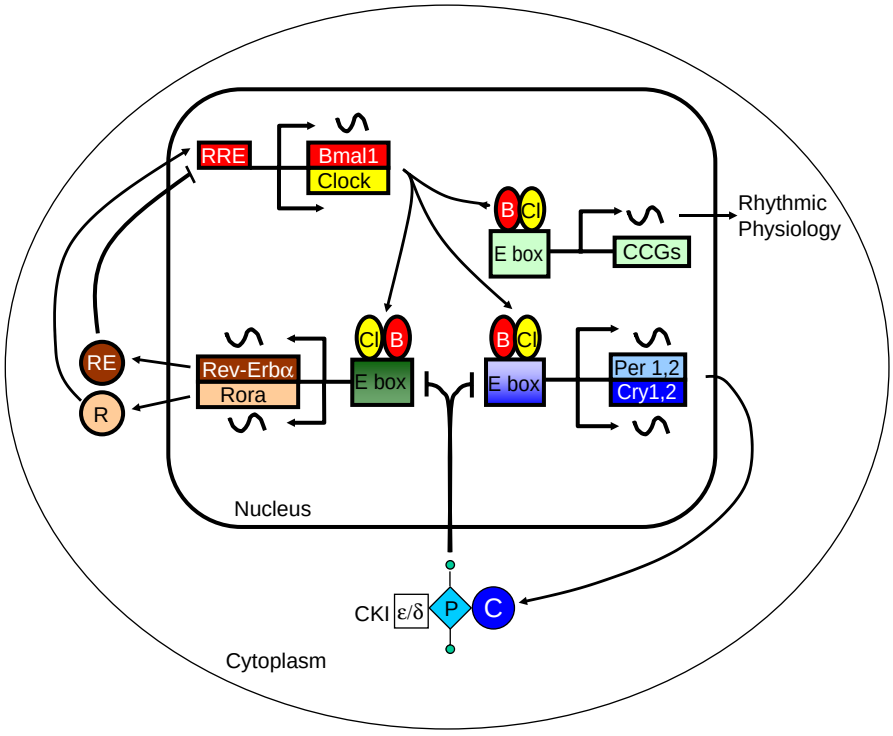

Fig. 1. Circadian clockwork of the mammalian SCN. Circadian clocks in a wide range of organisms are composed of two interdependent transcription–translation feedback loops that drive the periodic rhythms in the mRNA and protein levels of the clock components. In mammalian SCN, the first loop involves two bHLH-PAS-containing transcription factors, CLOCK (Cl) and BMAL1 (B). These transcription factors heterodimerize and activate the rhythmic transcription of three *period* genes (*Per1–Per3*, with *Per1* and *Per2* being critical to the circadian clock) and two *cryptochrome* genes (*Cry1* and *Cry2*). The PER (P) and CRY (C) proteins complex with casein kinase 1 δ and ε (CKIδ/ε), which phosphorylates PER. The resulting complex inhibits CLOCK/BMAL1-mediated transcription of *period* and *cryptochrome* genes, thus providing the negative feedback loop. The second loop involves CLOCK/BMAL1 driven rhythmic transcription of *Rev – erbα* and *Rora*, members of the retinoic acid-related orphan nuclear receptors. The phase of *Rora* expression closely resembles those of *Per1* and *Per2*, and is opposite in phase with *Rev – erbα*. The resultant REV – ERBα and RORa proteins (RE and R, respectively) compete for the same promoter element, Rev-erb/Ror element (RRE) and drive the rhythm in *Bmal1* transcription. CLOCK/BMAL1 heterodimers also bind to circadian E-boxes in clock-controlled genes (CCGs), providing an output from the clock that drives rhythmic physiology.

peripheral tissues, as rhythms persist in isolated tissues and cultured cells (Balsalobre et al., 1998; Yamazaki et al., 2000; Yoo et al., 2004). In PERIOD2:luciferase knock-in mice, SCN lesions do not eliminate circadian *Per2* reporter activity but cause phase de-synchrony among tissues and from animal to animal (Yoo et al., 2004).

1.3. Clocks in the retina

The retina is a remarkably rhythmic tissue. Many cellular, biochemical, and physiological processes change in a circadian fashion (Table 1), including visual sensitivity; rod outer segment disc shedding and phagocytosis by the retinal pigment epithelium (RPE); expression of immediate early genes and visual pigment genes in photoreceptors; second messenger levels and activities of enzymes in signal transduction pathways; expression of arylalkylamine *N*-acetyltransferase (AA-NAT) and the release of the neurohormone melatonin from photoreceptor cells; and release of the neuromodulator dopamine from inner retinal neurons.

Although each of the above rhythms may not occur in all vertebrate classes, there is overwhelming evidence that circadian rhythmicity is a highly conserved theme in retinal physiology. It is generally accepted that these retinal circadian rhythms allow the organism to anticipate and adapt to the >1 million-fold change in light intensity during a 24 h period, thereby optimizing visual function for each photic situation.

Nearly a quarter century has past since publication of the first definitive evidence for a circadian clock in the vertebrate eye, describing a daily rhythm in the activity of AANAT in isolated, cultured eye cups prepared from the African clawed frog (*Xenopus laevis*) (Besharse and Iuvone, 1983). AANAT is a key regulatory enzyme in the melatonin biosynthetic pathway. The AANAT rhythm occurs in retinas cultured under a daily light–dark cycle and persists for at least 3 days in constant darkness, indicative of rhythm generation by an intrinsic circadian clock. This rhythm drives a similar rhythm in melatonin release; the clock controlling these rhythms is located in photoreceptor cells (Cahill and Besharse, 1993).

Table 1
Circadian rhythms in the retina

Visual sensitivity and ERG responses[a]
Rod–cone dominance[b]
Cone photoreceptor retinomotor movements[c]
cAMP, Ras, B-Raf, ERK, and pCREB signaling pathways in photoreceptors[d]
Affinity of cyclic nucleotide-gated channels for cGMP[e]
Sensitivity to light-induced photoreceptor degeneration[f]
Transducin mRNA[g]
Iodopsin mRNA[h]
Melanopsin mRNA[i]
Nocturnin mRNA[j]
Spinule formation at cone-horizontal cell synapses[k]
Extracellular pH and energy metabolism[l]
Phospholipid metabolism in photoreceptor and ganglion cells[m]
Melatonin biosynthesis in photoreceptor cells[n]
Dopamine content and release from amacrine and interplexiform cells[o]

Selected references:
[a]Bassi and Powers (1987), Kurusu et al. (1993), Lu et al. (1995), Li and Dowling (1998), Ren and Li (2004), and Solessio et al. (2004).
[b]Wang and Mangel (1996) and Manglapus et al. (1998).
[c]Welsh and Osborne (1937), Burnside and Ackland (1984), and Pierce and Besharse (1985).
[d]Ko et al. (2001, 2004b), Liu and Green (2002), Ivanova and Iuvone (2003a), and Fukuhara et al. (2004).
[e]Ko et al. (2001).
[f]Organisciak et al. (2000) and Vaughan et al. (2002).
[g]Brann and Cohen (1987).
[h]Pierce et al. (1993).
[i]Chaurasia et al. (2005) and Sakamoto et al. (2004b).
[j]Green and Besharse (1996a) and Wang et al. (2001).
[k]Wagner et al. (1992) and Behrens et al. (2000).
[l]Dmitriev and Mangel (2000, 2001, 2004).
[m]Guido et al. (2001).
[n]Hamm and Menaker (1980), Cahill and Besharse (1993), Tosini and Menaker (1996), and Adachi et al. (1998).
[o]Ribelayga et al. (2002) and Doyle et al. (2002a).

Circadian clocks are known to be present in the retinas of many vertebrates (Thomas et al., 1993; Pierce et al., 1993; Tosini and Menaker, 1996, 1998; Zaunreiter et al., 1998; Ivanova and Iuvone, 2003a, b). Although these oscillators can be considered peripheral clocks, studies on rodents indicate they function in the absence of the SCN. For example, circadian rhythms of photoreceptor disc shedding and *AANAT* mRNA persist in SCN-lesioned rats (Terman et al., 1993; Sakamoto et al., 2000). Moreover, retinal clocks may influence the SCN, as enucleation disrupts rhythms of p44/p42 mitogen-activated protein kinase (MAPK) phosphorylation (Lee et al., 2003) and photoreceptor degeneration delays the nighttime increase of phosphorylation of the cAMP-response element binding protein (CREB) in the master clock (Alvarez-Lopez et al., 2004); these effects may not simply reflect removal of light/dark cues, but may also reflect clock-driven functions in the retina. In addition, enucleation lengthens the free-running period of behavioral rhythms compared to those observed in intact animals kept in constant darkness (Yamazaki et al., 2002). Thus, the circadian clock in the vertebrate eye may contribute to system-level circadian organization through effects on the SCN.

This review will describe recent progress in understanding circadian clock-controlled and light-regulated AANAT activity and melatonin synthesis in the chicken and rodent retina and will discuss emerging evidence for the existence of clocks, or clock networks, in multiple cell types in the retina.

2. AANAT, melatonin, and melatonin receptors in retina

Melatonin is synthesized from tryptophan by the following pathway:

tryptophan
↓ tryptophan hydroxylase (TPH)
5-hydroxytryptophan
↓ aromatic amino acid decarboxylase (AADC)
Serotonin
↓ arylalkylamine *N*-acetyltransferase (AANAT)
N-Acetylserotonin
↓ hydroxyindole *O*-methyltransferase (HIOMT)
melatonin.

Melatonin synthesis occurs in the pineal gland and—in many vertebrates—in the retina. Generally, circulating melatonin reflects production in the pineal gland (Lewy et al., 1977); however, in some avian species, the eyes can contribute significantly to circulating levels of the hormone (Underwood et al., 1984; Oshima et al., 1989). Synthesis of melatonin is significantly lower in most vertebrate retinas as compared to that in the pineal gland. Moreover, studies on human and primate retinas indicate that the last enzyme in the melatonin pathway appears to be absent, indicating that melatonin is not produced at significant levels in these tissues. In those retinas which synthesize melatonin, it is thought to act locally as a neuromodulator (Zawilska and Nowak, 1992; Iuvone and Alonso-Gómez, 1998). Studies on amphibians, birds, and rodent retinas indicate that melatonin synthesis exhibits circadian rhythmicity. In general, it is highest at night in darkness and suppressed by light, and melatonin is thought to promote dark-adaptive physiology in the retina (Besharse et al., 1988). However, studies in some fishes, notably trout and pike, indicate this pattern is reversed or shifted, i.e., that melatonin is high during most or all of the day (Gern and Ralph, 1979; Falcón and Collin, 1991; Zachmann et al., 1992; Iigo et al., 1997; Zaunreiter et al., 1998; Garcia-Allegue et al., 2001). In other fishes, such as the goldfish, melatonin levels in the retina are high at night (Iigo et al., 1997), as in other vertebrate species.

2.1. Melatonin production and AANAT

Studies in both the pineal gland and retina indicate that there is a highly conserved relationship between melatonin synthesis and AANAT activity when HIOMT is present. AANAT activity is greatly enhanced at night in both tissues, driving the rhythm of melatonin synthesis (Klein et al., 1999). In the case of the pineal gland, elevation of AANAT activity also increases *N*-acetylsertonin by >10-fold (Mefford et al., 1983), which drives melatonin production by a substrate availability mechanism. Light exposure at night reverses these effects.

Although AANAT expression is a conserved feature of the vertebrate retina, as indicated above, the synthesis of melatonin is not because HIOMT may be absent. For example, analyses of rhesus monkey and human retinas indicate that HIOMT protein and activity are undetectable and that *HIOMT* mRNA is detectable only by the most highly sensitive methodology (Rodriguez et al., 1994; Bernard et al., 1995; Coon et al., 2002). In contrast, high levels and daily rhythms of AANAT activity are seen in the rhesus monkey retina (Coon et al., 2002). The absence of HIOMT in the primate retina has led to speculation that circulating melatonin (Osol and Schwartz, 1984; Leino, 1984) or locally synthesized *N*-acetylserotonin may substitute for melatonin as a local signal (Nash and Osborne, 1995; Reppert et al., 1995a; Scher et al., 2002, 2003; Savaskan et al., 2002). In addition, it has been proposed that AANAT may play a conserved detoxification role in the vertebrate retina by broadly acetylating arylalkylamines in addition to serotonin (i.e., phenylethylamine and tryptamine), thereby preventing toxic reactions (Klein, 2004). Detoxification has been considered to be the original function of AANAT when first acquired by the primitive photoreceptor.

2.2. Sites of melatonin synthesis in the retina

Retinal melatonin synthesis occurs primarily in photoreceptors. This is indicated by the observations that melatonin and/or melatonin-synthesizing enzymes are retained in the retina following removal or lesions of the inner retina that leave photoreceptors intact (Zawilska and Iuvone, 1992; Cahill and Besharse, 1992; Thomas et al., 1993; Sakamoto et al., 2004a). Moreover, isolated photoreceptor layers from *Xenopus* retina rhythmically secrete melatonin, and light pulses can shift the phase of the rhythm of melatonin release from these isolated cells (Cahill and Besharse, 1993).

These observations indicate that photoreceptors contain a complete circadian system, with a circadian clock, a photoentrainment pathway, and an output pathway. In this regard, they are similar to the photosensitive pinealocyte from birds and some fish. The presence of AANAT in photoreceptor cells is supported by in situ hybridization histochemistry, which has identified *AANAT* mRNA in photoreceptors of chickens (Bernard et al., 1997), rats (Niki et al., 1998; Liu et al., 2004), and monkeys (Coon et al., 2002). In addition, *AANAT* mRNA has also been observed at distinctly lower levels in the inner nuclear layer and ganglion cell layer, suggesting that other cell types may possess limited capacity to synthesize *N*-acetylsertonin, melatonin, and/or other acetylated arylalkylamines (Bernard et al., 1997; Coon et al., 2002; Liu et al., 2004; Sakamoto et al., 2004a; Garbarino-Pico et al., 2004b).

2.3. Clock regulation of retinal melatonin production

The dramatic nocturnal increase of melatonin biosynthesis seen in chicken and *Xenopus* retinas is driven by clock-controlled increases in the activities of TPH and AANAT (Besharse and Iuvone, 1983; Cahill and Besharse, 1990; Thomas and Iuvone, 1991; Green and Besharse, 1994; Bernard et al., 1997; Chong et al., 1998; Thomas et al., 1998; Iuvone et al., 1999). In many species, both enzymes are encoded by CCGs whose expression is highly rhythmic (e.g., Green and Besharse, 1994; Bernard et al., 1997; Chong et al., 1998; Chen and Baler, 2000). In other species, circadian expression of one or both of these enzymes is not seen and rhythmic melatonin biosynthesis is generated by posttranscriptional mechanisms (Klein et al., 1999; Coon et al., 2002).

2.4. Melatonin signal transduction

2.4.1. G protein-coupled melatonin receptors

Melatonin acts primarily through 2–3 different subtypes of G protein-coupled (GPC) receptors, depending on species. These are designated Mel 1a (MT1), Mel 1b (MT2), and Mel 1c (Dubocovich et al., 2003). The retina expresses these receptors in most, if not all vertebrate species (Reppert et al., 1995a, b), suggesting that a major role for melatonin receptors in retinal function has been conserved throughout vertebrate evolution. However, the specific nature of this function is not clear.

Melatonin receptors have a high affinity for melatonin (K_D in the pM range) and a high degree of structural selectivity (Dubocovich and Takahashi, 1987). This high affinity allows melatonin receptors in many tissues to detect changes in circulating levels of melatonin. Melatonin receptors also bind *N*-acetylserotonin, albeit with significantly lower affinity than that for melatonin; this is the basis for considering it as a substitute for melatonin. Hence, in the context of the retina, melatonin receptors may physiologically function to mediate effects of circulating and locally generated melatonin and of locally generated *N*-acetylserotonin.

2.4.2. Quinone reductase 2

Melatonin and *N*-acetylserotonin may also act through the *MT3* binding site, which is not a GPC receptor. This site has a higher affinity for *N*-acetylserotonin than for melatonin (Dubocovich, 1995; Dubocovich et al., 2003) and has been tentatively identified as a melatonin-sensitive quinone reductase 2 (Nosjean et al., 2000), related to the detoxification enzyme quinone reductase 1. *MT3* is found in a variety of mammalian tissues, including brain (Nosjean et al., 2001). Localization of *MT3* in the eye has not yet been investigated, but a putative *MT3* ligand, 5-methoxycarbonylamino-*N*-acetyltryptamine, has been reported to decrease intraocular pressure in rabbit and monkey eyes (Pintor et al., 2003; Serle et al., 2004).

2.5. Effects of melatonin in the retina

GPC melatonin receptors occur on many retinal cell types, including RPE, photoreceptors, amacrine cells, and ganglion cells. Melatonin receptors on retinal neurons and RPE cells are negatively coupled to adenylyl cyclase (Iuvone and Gan, 1994; Nash and Osborne, 1995). Activation of melatonin receptors elicits effects in both the outer and inner retina, many of which suggest a role for melatonin as a chemical mediator of dark-adaptation in the retinal network (Besharse et al., 1988).

In the outer retina, melatonin causes dark-adaptive pigment migration in fish and guinea pigs (Cheze and Ali, 1976; Pang and Yew, 1979) and alters light-evoked electrical activity of the chicken RPE (Nao-I et al., 1989; Li et al., 1990; Rudolf et al., 1992). Melatonin alters porphyrin synthesis in bovine RPE in a light-dependent manner (Durkó et al., 1992) and inhibits the proliferation of cultured bovine RPE cells (Yu et al., 1993). It protects human RPE cells from oxidative stress (Liang et al., 2004) and from ischemia-induced apoptosis (Osborne et al., 1998). Melatonin activates rod outer segment disc shedding in retinas of *Xenopus* and rat (Besharse and Dunis, 1983; Besharse et al., 1984; White and Fisher, 1989), supporting a role for melatonin receptors in the circadian regulation of outer segment membrane turnover. However, melatonin is not essential for circadian disc shedding and phagocytosis in mice (Grace et al., 1999). Melatonin also promotes dark-adaptive cone photoreceptor elongation in *Xenopus* retina (Pierce and Besharse, 1985). It hyperpolarizes and increases the response of salamander horizontal cells to light (Wiechmann et al., 1988), mimicking effects of dark-adaptation at the photoreceptor to horizontal cell synapse. Melatonin sensitizes the rat retina to light-induced photoreceptor degeneration (Wiechmann and O'Steen, 1992), whereas the melatonin receptor antagonist luzindole decreases light damage (Sugawara et al., 1998).

In the inner retina of mammals and non-mammalian vertebrates, melatonin inhibits the release of dopamine (Dubocovich, 1983; Dubocovich and Takahashi, 1987; Boatright et al., 1994; Adachi et al., 1999; Ribelayga et al., 2004) and acetylcholine (Mitchell and Redburn, 1991), transmitters whose release is stimulated by light. In contrast, melatonin increases the release of glutamate (Faillace et al., 1996). Thus, melatonin and, possibly, *N*-acetylserotonin released from photoreceptors may diffuse throughout the retina to modulate neurotransmission at all levels. The effect of these compounds on dopamine release appears to be particularly important, as dopamine is a mediator of light adaptation (Nir et al., 2002) and circadian changes in visual sensitivity (Witkovsky et al., 1989; Wang and Mangel, 1996; Manglapus et al., 1999; Ribelayga et al., 2004). In addition, melatonin receptors are found on AII amacrine cells (Scher et al., 2003), which are interneurons in the rod pathway, which operates in low light.

3. Hypothetical model for circadian clock- and light-regulated AANAT activity in photoreceptor cells

The cellular and molecular mechanisms controlling AANAT expression and activity in the retinas of chicken, *Xenopus*, and rat is described in detail below; the hypotheses presented are based primarily on research using these species. In addition, some of the mechanistic similarities and differences in other species are addressed.

Interest in understanding the regulation of melatonin synthesis has led investigators to concentrate on AANAT because of the critical regulatory role it plays in melatonin synthesis in the pineal gland and in most retinas and because it exhibits large changes in activity. In essentially all species, AANAT activity is elevated at night and rapidly inhibited by light in both the retina and pineal gland (Klein et al., 1997), which insures that the activity of this enzyme is an accurate reflection of the photoperiod. The circadian and light-evoked fluctuations of retinal and pineal melatonin biosynthesis are driven largely by changes in the activity of AANAT (Klein et al., 1997; Iuvone et al., 1999). However, a notable exception exists in trout and other salmonids; in these species, retinal AANAT activity and melatonin synthesis are elevated during the day, opposite to the pattern in the pineal gland and other retinas (Gern and Ralph, 1979; Falcón and Collin, 1991; Zachmann et al., 1992; Iigo et al., 1997; Zaunreiter et al., 1998; Garcia-Allegue et al., 2001).

Key aspects of AANAT activity regulation in the chicken retina are depicted in Fig. 2. In retinas exposed to a light–dark cycle, AANAT activity is low during the daytime, increases rapidly early in the night, and begins

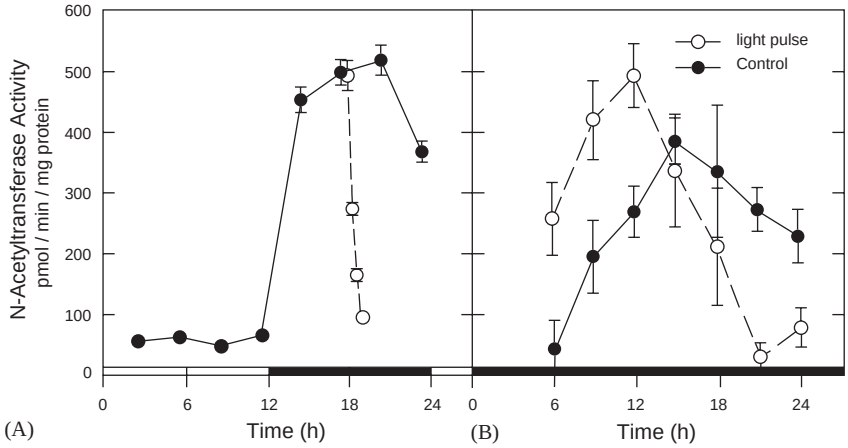

Fig. 2. Daily rhythm of retinal AANAT activity: effects of light. (A) AANAT activity fluctuates during the 12 h light–12 h dark cycle (filled circles). Unexpected light exposure at night (open circles) rapidly inhibits activity. (B) AANAT activity in constant (24 h/day) darkness. The activity rhythm persists on the second day of constant darkness (filled circles). The rhythm is phase advanced by a 6 h light pulse from 1800 to 2400 h 2 days prior to sampling in constant darkness (open circles). Filled bars on the *x*-axis represent darkness; open bars represent light. Activity was measured in retinal homogenates of 2-week-old chickens. Reproduced from Iuvone and Alonso-Gómez (1998); © Editions IRVINN.

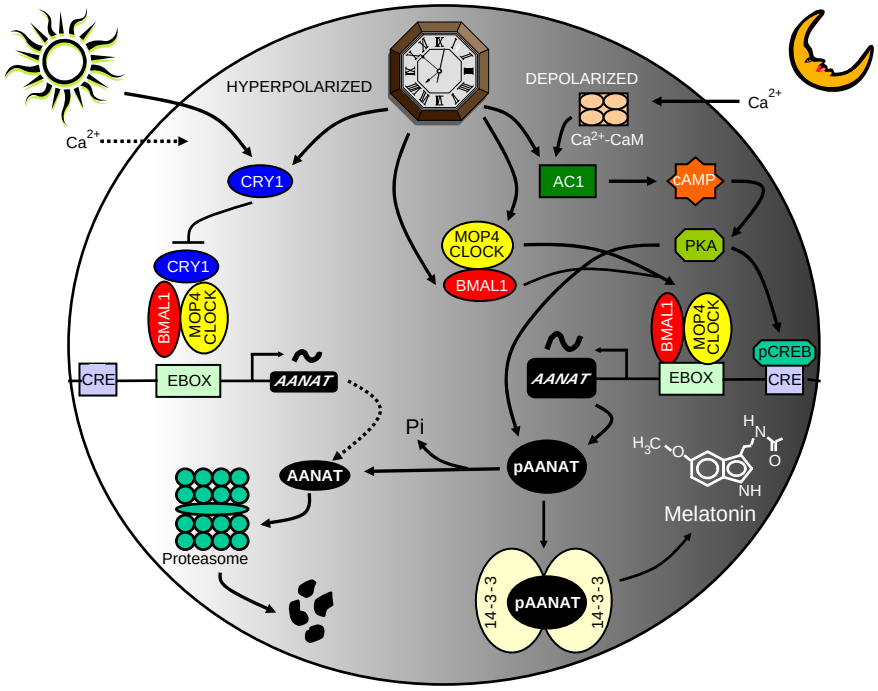

Fig. 3. Hypothetical model for circadian-clock and light-regulated melatonin biosynthesis in photoreceptor cells. See text for detailed description of the model. The left side of the figure depicts processes occurring in light while the right side shows processes occurring at night in darkness.

to decline in anticipation of dawn (Fig. 2A). The rhythmic expression of AANAT activity persists in constant darkness (Fig. 2B). Light has two effects on AANAT activity. Exposure to light in the middle of night rapidly decreases AANAT activity, with a half-life of ~20 min (Fig. 2A). This occurs without any corresponding change in *AANAT* mRNA (Bernard et al., 1997). Light also shifts the phase of the AANAT activity rhythm (Fig. 2B).

The following hypothetical model describes clock-controlled and light-driven regulation of AANAT activity and melatonin synthesis (Fig. 3). A circadian clock in the photoreceptor cell is entrained by the environmental light–dark cycle via a novel, transducin-independent transduction mechanism. The clock generates a rhythm of *AANAT* gene transcription by interactions of circadian clock proteins, BMAL:-CLOCK/MOP4, with an E-box enhancer element in

its promoter. This leads to a circadian rhythm of *AANAT* mRNA and protein, which are both elevated at night. In darkness, photoreceptors are depolarized, allowing Ca^{2+} influx and stimulating cAMP formation by a Ca^{2+}-dependent adenylyl cyclase (AC1 and/or AC8). The Ca^{2+}-evoked stimulation of cAMP is gated by the circadian clock, which controls the expression of *AC1*, leading to a light-sensitive circadian rhythm of cAMP, with peak levels of cAMP at night. cAMP-dependent phosphorylation of transcription factors augments the E-box-driven increase of *AANAT* mRNA and protein. cAMP-dependent protein kinase (PKA) also phosphorylates AANAT, which stabilizes it in an active state through an interaction with 14-3-3 proteins, resulting in an increase in melatonin synthesis and release. Upon exposure to light, photoreceptors hyperpolarize, leading to decreased intracellular Ca^{2+} and cAMP. These events result in enzyme dephosphorylation, dissociation of AANAT/14-3-3 complexes with a concomitant decrease in affinity for substrate, rapid proteolysis of the enzyme by proteasomes, and, consequently, rapid decreases of melatonin synthesis. A discussion of the evidence supporting each step of this model follows.

3.1. Circadian clock entrainment

A circadian clock in the photoreceptor cell is entrained by the environmental light–dark cycle via a novel, transducin-independent transduction mechanism. The evidence for this occurring in retinal photoreceptor cells is largely indirect and based mostly on studies with chicken pineal photoreceptors. Melatonin synthesis in these cells show the same two responses to light as observed in retina; light exposure at night acutely inhibits melatonin synthesis and phase shifts the circadian rhythm of melatonin synthesis on subsequent days (Zatz and Mullen, 1988). The mechanism responsible for shifting the phase of the circadian oscillator by light differs from that involved in light-evoked suppression of melatonin biosynthesis. Pre-treating pinealocytes with pertussis toxin, which ADP-ribosylates and inactivates transducin (G_T) and other select G proteins, blocks the acute suppression of melatonin biosynthesis by light but not the light-evoked shifts of circadian phase (Zatz and Mullen, 1988). Suppression of melatonin synthesis in photosensitive pinealocytes by light appears to be mediated by G_T (Kasahara et al., 2000), while phase shifting involves a pertussis toxin-insensitive, transducin-independent signaling pathway. The effect of light on phase shifting may involve another G protein, which is not affected by pertussis toxin, or a G protein-independent pathway. Pinealocytes and photoreceptors express $G_{\alpha 11}$ (Peng et al., 1997; Kasahara et al., 2002), a pertussis toxin-insensitive G protein. Opsin activates GTP exchange by $G_{\alpha 11}$, and the coupling of $G_{\alpha 11}$ to phospholipase C has been implicated in phase shifting in chick pinealocytes (Kasahara et al., 2002). It is unknown if a similar relationship exists in retinal photoreceptors. There is suggestive evidence that photoentrainment of the retinal photoreceptor clock also involves a novel signaling mechanism. Light appears to entrain photoreceptor circadian oscillators in the retina of the rd chicken (Larkin et al., 1999), which has a null mutation in guanylyl cyclase 1 and, consequently, no 'classical' rod and cone visual transduction cascades.

The mechanisms for entrainment of the photoreceptor circadian clock by light and darkness are poorly understood. Short periods of light at night or darkness during the daytime can phase shift or re-entrain the circadian oscillator (Cahill and Hasegawa, 1997). In *Xenopus* photoreceptor layers, depolarizing concentrations of extracellular K^+ or cAMP protagonists mimic the effect of darkness on circadian phase (Hasegawa and Cahill, 1998, 2004a). However, the phase-shifting effects of light in this preparation do not appear to be attributable to decreases of cAMP (Hasegawa and Cahill, 1999). The effects of light on circadian phase are attenuated by an inhibitor of the stress-activated p38 MAPK and Jun N-terminal kinase (JNK) (Hasegawa and Cahill, 2004b). These studies implicate cAMP and MAPK signaling pathways in clock entrainment, but the mechanisms are still unknown.

3.2. Clock genes and AANAT gene transcription

The clock generates a rhythm of AANAT gene transcription by interactions of circadian clock proteins, BMAL:CLOCK/MOP4, with an E-box enhancer element in its promoter, and the mRNA is translated into enzyme protein. Evidence supporting this comes from studies of the chicken retina, which expresses a circadian rhythm of *AANAT mRNA* (Fig. 4); this is quickly translated into AANAT protein and activity (Bernard et al., 1997; Iuvone et al., 2002). The *AANAT* promoter contains a circadian E-box enhancer element (Chong et al., 2000), which may provide a link from the clock to *AANAT* transcription (Fig. 3). The circadian clock components, BMAL1 and CLOCK, heterodimerize and activate E-box-mediated transcription of other clock genes, such as the *Per* genes (Reppert and Weaver, 2002) (Fig. 1). Co-transfection of COS7 cells with BMAL1 and CLOCK activates an *AANAT*-E-box-luciferase reporter (Chong et al., 2000). Interestingly, another bHLH-PAS protein, MOP4 (also called NPAS2), which is found in mammalian forebrain but not in the central clock mechanism of the SCN (Shearman et al., 1999; Reick et al., 2001), was also capable of activating the *AANAT* E-box when co-expressed with BMAL1. MOP4 is required for rhythmic *Per* expression in mouse forebrain (Reick et al., 2001). These studies suggest that

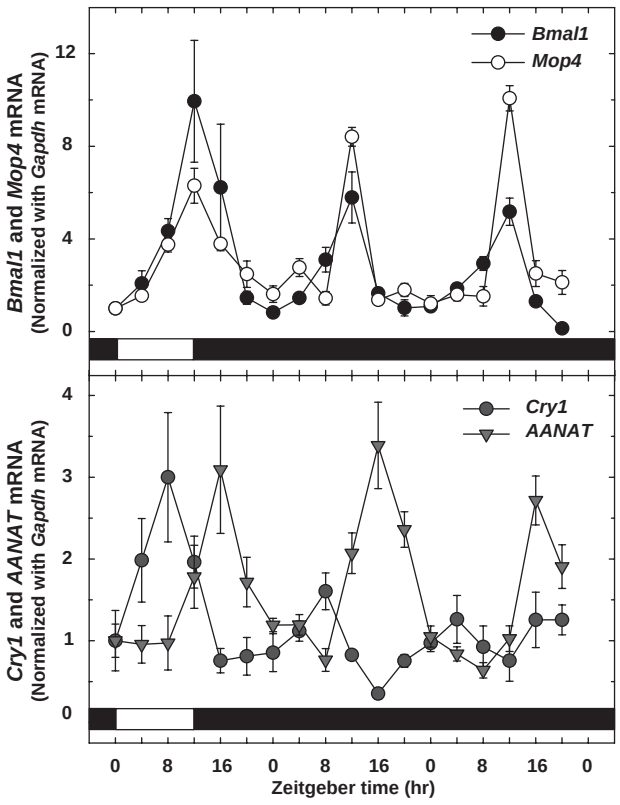

Fig. 4. Rhythmic expression of circadian clock genes and *AANAT* in avian retina. One-day-old chicks were housed in a 12 h light:12 h dark (LD) cycle with lights on at zeitgeber time (ZT) 0 for 2 weeks and later kept under constant darkness (DD) for 2 days. Tissues were collected in LD and DD at ZT 0, 4, 8, 12, 16, and 20. Relative mRNA levels in the tissues were quantified by real-time RT-PCR. *Bmal1*, *Mop4*, *Cry1*, or *AANAT* transcript levels were normalized to *Gapdh* mRNA and expressed relative to the ZT 0 values in LD. The open bars indicate the times of lights-on and the filled bars indicate times of darkness. Rhythmic expression of all genes was observed in LD. Rhythmic expression of *AANAT*, *Bmal1*, and *Mop4* was observed in DD, while the rhythm of *Cry1* became damped in constant darkness. Rhythms of all genes, including *Cry1*, persisted in constant light (data not shown; Haque et al., 2002). Data replotted from Chong et al. (2003; *Bmal1*, *Mop4*) and Haque et al. (2002; Cry1, AANAT).

components of the circadian clock and related transcription factors directly activate the promoter of this CCG. Expression of dominant-negative CLOCK in *Xenopus* photoreceptor cells abolished the circadian rhythm of melatonin synthesis without affecting the levels of its production (Hayasaka et al., 2002). It is unclear at present whether this effect is due specifically to loss of *AANAT* transcriptional regulation or to a more generalized disruption of circadian clock function in photoreceptors.

Transcripts for several clock genes are rhythmically expressed in chicken retina (Fig. 4). *Bmal1* and *Mop4* transcripts peak together at zeitgeber time (ZT) 12, approximately 4 h prior to the peak of *AANAT* mRNA. This timing is consistent with the hypothesis that bHLH-PAS proteins drive the *AANAT* rhythm (Fig. 3). The *Cry1* mRNA rhythm is 8 h out of phase with the *AANAT* transcript rhythm, peaking at ZT 8 (Fig. 4). CRY1 inhibits BMAL/CLOCK-mediated activation of the E-box promoter element (Griffin Jr. et al., 1999; Yamamoto et al., 2001) and this timing is consistent with a possible role of CRY1 in inhibition of *AANAT* transcription in the daytime (Fig. 3). *Cry2* and *Clock* transcripts are also expressed in chicken retina (Larkin et al., 1999; Bailey et al., 2002). Larkin et al. (1999) reported a low amplitude rhythm of *Clock* mRNA in chicken retina. The temporal pattern of *Cry2* mRNA expression in retina is unknown.

Similar mechanisms appear to be operative in other vertebrate species. In rat and mouse retinas, rhythms of *AANAT* mRNA are translated into rhythms of AANAT protein and activity (Sakamoto and Ishida, 1998a, b; Fukuhara et al., 2001). The rat *AANAT* promoter contains an E-box and co-transfection of BMAL1 and CLOCK in rat retinal cells, but not pineal cells, activates the *AANAT* promoter-luciferase reporter (Chen and Baler, 2000; Tosini and Fukuhara, 2002). In the monkey, *AANA*T mRNA is not rhythmic, but AANAT activity is (Coon et al., 2002).

Other clock genes have been identified in retina. In the eye of the Japanese quail, which also contains a clock that regulates melatonin biosynthesis (Underwood et al., 1990), *Per2* and *Per3* have been identified (Yoshimura et al., 2000). Transcripts of both genes are rhythmically expressed, with highest levels in the beginning of the subjective day. Quail *Per2*, but not *Per3*, is induced by light exposure and may be involved in photoentrainment of the circadian clock (Yoshimura et al., 2000). *Xenopus* photoreceptors express *Per1* and *Per2* (Besharse et al., 2004). Transcripts of both genes show diurnal rhythms, but with opposite phase relationships (Zhuang et al., 2000). *Per1* expression is highest at dawn, while *Per2* transcript levels are highest late in the day. The rhythm of *Per1* expression is maintained in constant darkness, while that of *Per2* is light-driven. As in the quail retina, the induction of *Per2* by light is thought to play a role in entrainment (Zhuang et al., 2000; Besharse et al., 2004).

Some studies have investigated the pattern of expression of the clock genes in the mammalian retina. *Per* genes are rhythmically expressed in the retina of the mouse where they peak at ZT 12, ~6 h later than in the SCN (Sun et al., 1997). *Clock*, *Cry1*, and *Cry2* are expressed in the mouse retina but in an arrhythmic manner (King et al., 1997; Miyamoto and Sancar, 1998). *Per2* is rhythmically expressed in the rat retina with a peak around ZT 15–18 (Oishi et al., 1998; Sakamoto et al., 2000; Namihira et al., 2001), while *Per1* mRNA is apparently not rhythmic (Namihira et al., 2001). *Clock* and *Bmal1* mRNAs have been also detected in the rat retina, but the results obtained are contradictory. Oishi et al. (1998) reported that *Bmal1* mRNA is rhythmic, whereas Namihira et al. (1999) were unable to detect a

rhythm. How the expression of these clock genes relates to melatonin synthesis in the mammalian retina is presently unknown.

3.3. Ca^{2+}, cAMP, and AANAT activity

Calcium influx in darkness stimulates cAMP formation and AANAT activity in photoreceptor cells. cAMP levels in photoreceptors are high in darkness and reduced by exposure to light (Orr et al., 1976; Farber et al., 1981; Cohen, 1982; Burnside et al., 1982; Denton et al., 1992). cAMP analogs and forskolin, an adenylyl cyclase activator, stimulate AANAT activity in light-exposed retinas of a variety of vertebrates species (Iuvone and Besharse, 1983, 1986c; Iuvone, 1990; Nowak, 1990) and in cultured cells (Iuvone et al., 1990; Wiechmann, 1996). cAMP increases *AANAT* mRNA abundance in chick retina and inhibits the inactivation of the enzyme by a proteolytic mechanism (Alonso-Gómez and Iuvone, 1995a; Iuvone et al., 1997).

The dark-dependent increase of AANAT activity and melatonin synthesis requires Ca^{2+} influx through dihydropyridine-sensitive voltage-gated channels (Iuvone and Besharse, 1986a), which are on photoreceptor inner segments and terminals (Fain et al., 1980; Corey et al., 1984; Barnes and Hille, 1989; Avendano et al., 1990; Gleason et al., 1992). Photoreceptors are partially depolarized in darkness and are hyperpolarized by light (Werblin and Dowling, 1969; Hagins et al., 1970). Consequently, Ca^{2+} levels in photoreceptors are high in darkness and reduced by light exposure. Thus, hyperpolarization and inhibition of Ca^{2+} influx may mediate the suppression of AANAT activity by light. Inhibition of dihydropyridine-sensitive Ca^{2+} channels blocks the dark-dependent, nocturnal increase of AANAT activity and mimics the effect of light exposure in *Xenopus* retina in vitro (Iuvone and Besharse, 1986a) and in chick retinal photoreceptor cells in vivo and in vitro (Zawilska and Nowak, 1990; Ivanova and Iuvone, 2003b). The effect of Ca^{2+} is mediated, at least in part, by stimulation of cAMP formation. In photoreceptor-enriched chick retinal cell cultures, Ca^{2+} influx stimulates cAMP formation and AANAT activity, effects that are reversed by Ca^{2+} channel blockade (Avendano et al., 1990; Iuvone et al., 1991). The effects of Ca^{2+} influx on AANAT activity are also blocked by inhibitors of adenylyl cyclase and PKA (Gan et al., 1995). These data indicate the involvement of Ca^{2+} and cAMP in photic and circadian regulation of melatonin biosynthesis and, perhaps, other aspects of photoreceptor metabolism.

Ca^{2+} may elicit its effects on cAMP formation and AANAT activity through Ca^{2+}/calmodulin-stimulated adenylyl cyclases (Fig. 3). Avian and mammalian photoreceptors express the type 1 (AC1) and type 8 (AC8) adenylyl cyclases, which are activated by Ca^{2+}/calmodulin (Xia et al., 1993; Fukuhara et al., 2004; Iuvone et al., 2004). The stimulation of cAMP formation and AANAT activity by Ca^{2+} in cultured chick retinal cells is inhibited by calmodulin antagonists (Alonso-Gómez and Iuvone, 1995b; Iuvone et al., 1996).

Intracellular Ca^{2+} levels may be regulated by Ca^{2+} entry through several types of channels. As mentioned above, photoreceptors contain voltage-gated Ca^{2+} channels that have a high open probability when the plasma membrane is depolarized, as in darkness. In addition, Ca^{2+} influx occurs through cGMP-gated channels, which also have a high open probability in darkness (Yau and Baylor, 1989). In cultured chick retinal cells, cGMP analogs increase intracellular levels of Ca^{2+} and cAMP and the activity of AANAT (Haque and Iuvone, 1999).

The stimulation of intracellular Ca^{2+} and cAMP levels by cGMP analogs is only partially blocked by dihydropyridine Ca^{2+} channel blockers, but completely eliminated following Ca^{2+} chelation with EGTA. Thus, influx through the cGMP-gated channels may contribute significantly to the stimulation of intracellular Ca^{2+} in darkness. Another potential source of intracellular Ca^{2+} is a current referred to as I_{LOT}. I_{LOT} is a non-selective cation current with a long open time (LOT) expressed in chicken pinealocyte and photoreceptor membranes (D'Souza and Dryer, 1996; Dryer et al., 1997). Significantly, cGMP-gated channels and I_{LOT} are regulated in a circadian fashion. The affinity of the cGMP-gated channel for cGMP is higher at night than during the daytime (Ko et al., 2001) and I_{LOT} has a higher open probability at night than during the day (Dryer et al., 1997). Thus, Ca^{2+} influx via cGMP-gated and LOT channels may contribute to the circadian regulation of cAMP.

3.4. Circadian control of cAMP

The Ca^{2+}-evoked stimulation of cAMP is gated by the circadian clock, which controls the expression of AC1, leading to a light-sensitive circadian rhythm of cAMP, with peak levels of cAMP at night. cAMP-dependent phosphorylation of transcription factors augments the E-box-driven increase of AANAT mRNA. Support for this comes in part from studies with photoreceptor-enriched chick retinal cell cultures, which express a circadian rhythm of cAMP with peak levels at night that persists in constant darkness (Ivanova and Iuvone, 2003a). This rhythm, as well as the AANAT activity rhythm, is light-sensitive, as exposure to light at night reduces cAMP levels and AANAT activity to daytime values (Ivanova and Iuvone, 2003a, b).

A similar circadian rhythm of cAMP has been observed in rat retina (Fig. 5) where it appears to play an important role in gating AANAT activity and melatonin biosynthesis (Fukuhara et al., 2004). Exposing cultured rat retina to darkness at different times of

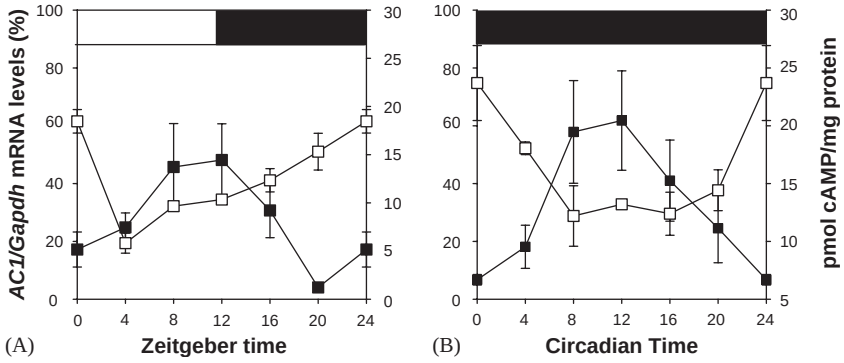

Fig. 5. Circadian regulation of *AC1* mRNA and cAMP levels in rat retina. (A) Retinal samples were collected under LD cycles at the times indicated. (B) Animals were first entrained to the LD cycle and then transferred to constant dim red light (<0.1 lux) for 3 days before sacrifice. The open bar at the top of the graph represents the period of white light, while the closed bars represent periods of darkness (A) or dim red light (B). *AC1* mRNA (black squares) and cAMP (white squares) levels were measured using real-time quantitative RT-PCR and radioimmunoassay, respectively. *AC1* mRNA abundance and cAMP levels were rhythmic in LD and DD, with the *AC1* transcript rhythm preceding that of cAMP by ~12 h. Reproduced from Fukuhara et al. (2004) © Society for Neuroscience.

the day stimulates melatonin synthesis only in the late afternoon and at night. Darkness has no effect in the morning, when cAMP levels are lowest. Forskolin, an activator of adenylyl cyclases, is also incapable of stimulating melatonin synthesis in the morning. In contrast, the PKA activator 8-CPT-cAMP stimulates melatonin synthesis at any time of the day. This finding suggests that adenylyl cyclase is a clock-controlled limiting factor for melatonin synthesis early in the day; this is supported by the existence of a circadian rhythm of *AC1* expression in rat (Fukuhara et al., 2004) and chick photoreceptors (Iuvone et al., 2004). As seen in Fig. 5, *AC1* mRNA increases during the daytime and is low at night. Consistent with the temporal delay expected for the processing of a membrane protein and the daily gating of melatonin synthesis, AC1-like immunoreactivity in photoreceptor inner segments and retinal cAMP levels are high during the nighttime and low during the day (Fukuhara et al., 2004).

AC1 appears to be a CCG. The proximal promoter of the *AC1* gene contains an E-box regulatory element (Chan et al., 2001). Transient transfection of rat retinal cells with BMAL/CLOCK stimulates *AC1* promoter-luciferase reporter activity (Fukuhara et al., 2004), suggesting that the *AC1* gene, like the *AANAT* gene, may be subject to direct transcriptional regulation by clock proteins. It should be noted, however, that the circadian rhythm of *AC1* mRNA peaks significantly earlier in the day than that of *AANAT* mRNA. This may be due to differential effects of cAMP on the two promoters. cAMP inhibits *AC1* transcription (Chan et al., 2001) but stimulates *AANAT* transcription via cAMP-response elements (CRE) in the *AANAT* promoter (Baler et al., 1997; Grève et al., 1999; Haque et al., 2004). Thus, the low levels of cAMP during the daytime may be permissive for BMAL/CLOCK-mediated activation of the *AC1* gene, while the high levels of cAMP at night may facilitate BMAL/CLOCK-mediated transactivation of the *AANAT* gene. These findings support a model in which the circadian clock regulates *AANAT* transcription by two mechanisms: a direct E-box-mediated activation in the *AANAT* promoter and an indirect CRE-mediated activation, resulting from enhanced *AC1* transcription, which leads to elevated cAMP and pCREB (Fig. 3).

Recently, downstream regulatory element antagonist modulator (DREAM)-response elements (DREs) have been identified in the rat *AANAT* promoter (Link et al., 2004). DREAM is a Ca^{2+}- and cAMP-sensitive transcriptional repressor (Carrion et al., 1999; Ledo et al., 2000). DREAM is expressed in retina and binding of nuclear extract proteins to the *AANAT*-DRE shows a diurnal rhythm, with highest binding during the daytime (Link et al., 2004). This temporally regulated binding is consistent with a role for DREAM in suppressing daytime *AANAT* expression. Moreover, DREAM represses transcription of an *AANAT* promoter in transfected cells. Whether functional DREs are present in the *AANAT* promoters of other species has not yet been reported.

3.5. Light, 14-3-3, and AANAT stability

cAMP-dependent protein kinase also phosphorylates the enzyme, which stabilizes it in an active state through an interaction with 14-3-3 proteins, resulting in an increase in melatonin synthesis and release. Upon exposure to light, photoreceptors hyperpolarize, leading to decreased intracellular Ca^{2+} and cAMP. These events result in enzyme dephosphorylation, dissociation of AANAT/14-3-3 complexes, rapid proteolysis of the enzyme, and, consequently, rapid decreases of melatonin release. The involvement of proteasomal proteolysis and 14-3-3 proteins in the control of AANAT was established by studies on the pineal gland (Gastel et al., 1998; Ganguly et al., 2001, 2004). The involvement of this

mechanism in control of retinal AANAT is based on findings that light exposure at night results in rapid inactivation and degradation of AANAT in the retina and in photoreceptor-enriched retinal cell cultures (Fukuhara et al., 2001; Iuvone et al., 2002; Ivanova and Iuvone, 2003b). High levels of cAMP, like those in darkness, stabilize the enzyme, preventing its inactivation (Alonso-Gómez and Iuvone, 1995a). Decreased levels of cAMP, as observed in light, result in a shortened half-life of the enzyme.

The mechanisms whereby cAMP stabilizes AANAT and light promotes its degradation are poorly understood. The rapid degradation of AANAT in retina and pineal gland in response to light exposure is blocked by proteasome inhibitors (Gastel et al., 1998; Zatz et al., 2000; Iuvone et al., 2002), implicating proteasomal proteolysis. AANAT is phosphorylated by PKA at two sites, one near the N-terminal and another near the C-terminal (Klein et al., 1997; Ganguly et al., 2001, 2004). While cAMP stabilizes and increases the half-life of the enzyme in photoreceptors (Alonso-Gómez and Iuvone, 1995a), phosphorylation of recombinant, purified GST-AANAT or cleaved AANAT has little effect on enzyme activity (Zhan-Poe and Craft, 1999; Ganguly et al., 2001).

3.5.1. Regulation of AANAT by binding to 14-3-3 proteins

The effect of cAMP on pineal AANAT is mediated through phosphorylation-dependent binding to 14-3-3 proteins (Ganguly et al., 2001). 14-3-3 proteins are a highly conserved family of chaperone proteins involved in signal transduction, regulation of the cell cycle, intracellular trafficking/targeting, cytoskeletal structure, and gene transcription (Aitken et al., 2002).

The N-terminal PKA phosphorylation site of AANAT is embedded in a consensus 14-3-3 binding motif (RRHpTLPAN) (Ganguly et al., 2001; Obsil et al., 2001; Aitken et al., 2002). AANAT and 14-3-3 interact in a phosphorylation-dependent manner. In addition, AANAT binds to 14-3-3 via the C-terminal PKA site, which may represent an additional consensus 14-3-3 binding motif found at the C-termini of proteins. Dual binding via both sites optimally positions the enzyme in the main channel of 14-3-3 so as to alter the conformation of a floppy loop of protein that is part of the arylalkylamine binding site. This induces higher affinity binding to serotonin and other arylalkylamine substrates; at approximate physiological concentrations of serotonin, this binding produces an ~10-fold increase in acetylation (Ganguly et al., 2004). Binding to 14-3-3 also protects it from proteolysis (Ganguly et al., 2001).

In addition, single-site binding via the C-terminal PKA site increases the Km for arylalkylamine substrates, thereby turning off acetylation of physiological concentrations. Accordingly, this residue mediates both activation and inactivation of AANAT (Ganguly et al., 2004).

The binding of AANAT to 14-3-3 is thought to be highly dynamic and the balance between free, dual-site bound, and single-site bound forms is determined by phosphorylation and dephosphorylation. Free AANAT is thought to be subject to proteolytic degradation whereas 14-3-3-bound AANAT is protected from destruction.

Interestingly, 14-3-3 also binds to TPH (Banik et al., 1997), the other regulated enzyme in the melatonin biosynthetic pathway, and may serve as a molecular scaffold to organize and stabilize a melatonin-synthesizing complex. 14-3-3 protein has been localized by immunocytochemistry to photoreceptor inner segments, where it apparently binds to phosducin in a phosphorylation-dependent, light-inhibited manner (Nakano et al., 2001; Thulin et al., 2001).

Our current working hypothesis is that AANAT in the retina is regulated as it is in the pineal gland, and that binding to 14-3-3 is favored when the enzyme is phosphorylated in the dark (Fig. 3). This interaction stabilizes the enzyme, protects it from degradation and increases the affinity for substrate. Upon light exposure, the enzyme dissociates from 14-3-3, is inactivated and dephosphorylated, and is targeted for degradation by the proteasome.

3.5.2. 14-3-3 control of AANAT in the retina

The above hypothesis is supported by recent results from studies on chicken retinas. Gel filtration chromatography of extracts of nighttime, dark-adapted chicken retinas, yields a major peak of activity with an apparent molecular weight of 70–90 kDa (Pozdeyev, Taylor, and Iuvone, unpubl. obs.), which approximates the molecular mass of AANAT (23 kDa) plus two molecules of 14-3-3 (~60 kDa). AANAT and 14-3-3 proteins co-immunoprecipitate from the fraction corresponding to 70–90 kDa peak of enzyme activity.

Following brief light exposure of retinas treated with lactacystin, a proteasomal protease inhibitor, the activity of AANAT/14-3-3 complex decreases and that of a low molecular weight form (~20kDa), corresponding to monomeric AANAT, increases. The monomeric enzyme has an apparent Km for substrate that is ~15-fold higher than that of the enzyme complexed with 14-3-3. Thus, AANAT in darkness exists primarily in a protein complex that dissociates upon light exposure. The high Km of the monomeric enzyme suggests that it has little activity in situ with physiological concentrations of substrate.

A third peak of retinal AANAT activity with an apparent molecular weight >150 kDa has been detected. The enzyme from this peak has a Km similar to that of monomeric AANAT and may represent the next step in the pathway of AANAT degradation

(ubiquitinated or docked to the proteasome). The significance of AANAT phosphorylation is supported by results showing that the treatment of protein extracts from light-exposed chicken retinas with PKA catalytic subunit causes re-association of monomeric AANAT into the AANAT/14-3-3 complex.

Another line of evidence comes from the effects of peptides containing the 14-3-3 binding motif fused to the protein transduction domain of the *Drosophila* antennapedia (AP) homeobox protein. The 16 amino acid AP peptide translocates efficiently across cell membranes by a receptor-independent mechanism (Derossi et al., 1994, 1996; Mi et al., 2000). We reasoned that if we could introduce into photoreceptors a peptide containing the AANAT sequence involved in 14-3-3 binding, this would interfere with AANAT/14-3-3 complex formation by a competitive mechanism.

Treatment with AP-AANAT$_{24-39}$ pT31 inhibits AANAT activity in cultured retinal cells in darkness with an IC_{50} of ~1 μM (Ivanova, Ganguly, Klein, and Iuvone, unpubl. obs.). Further studies are needed to confirm the mechanism of action of the AP-pAANAT$_{24-39}$ and to prove that 14-3-3 is the primary binding partner of AANAT in the 70–90 kDa complex.

4. Is cAMP a mediator of other circadian clock outputs in photoreceptor cells?

A circadian rhythm of cAMP suggests a generalized mechanism whereby the circadian clock can control multiple outputs (Fig. 6). Several aspects of circadian retinal physiology are influenced by cAMP. Rod photoreceptor disc shedding is inhibited by cAMP (Besharse et al., 1982). Dark-adaptive cone elongation and rod contraction in *Xenopus* and fish are stimulated by cAMP (Besharse et al., 1982; Burnside et al., 1982; Burnside and Ackland, 1984). These effects are temporally coincident with the rhythms of cAMP. Rod disc shedding is most robust in the morning when cAMP levels are lowest and is suppressed at night. Circadian cone elongation and rod contraction occur at night, when cAMP levels are high. Thus, the circadian rhythm of cAMP may drive the rhythms of adaptive physiology.

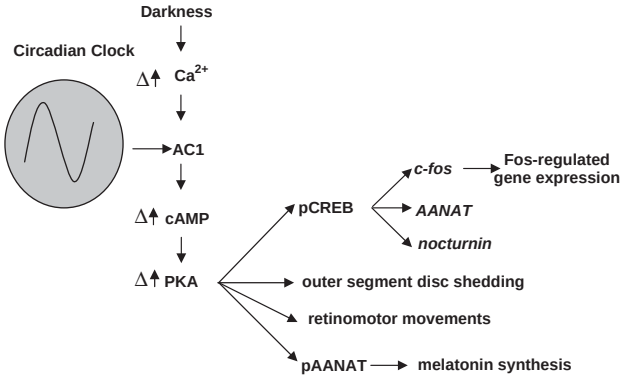

Fig. 6. cAMP: a pivotal regulator of circadian clock output in photoreceptor cells? The circadian clock regulates the expression of the Ca^{2+}/calmodulin sensitive adenylyl cyclase AC1, leading to a light-sensitive circadian rhythm of cAMP. AC1 is stimulated by Ca^{2+} and produces more cAMP in darkness, when photoreceptor cells are depolarized and Ca^{2+} levels are high, than in light. This light-sensitive rhythm of cAMP leads to light-sensitive rhythms of gene expression and photoreceptor cell physiology.

Nocturnin is an RNA deadenylase that is expressed in *Xenopus* photoreceptors and various mammalian tissues under the influence of the circadian clock (Green and Besharse, 1996a, b; Wang et al., 2001; Baggs and Green, 2003). The *nocturnin* gene shows a high amplitude rhythm of transcription in photoreceptors that peaks at night. The *nocturnin* promoter contains a CRE-like regulatory element that binds phosphorylated and unphosphorylated CREB (Liu and Green, 2002). Phosphorylated CREB activates *nocturnin* transcription and there is a circadian rhythm in the levels of phospho-CREB in photoreceptor cells that also peaks at night. Thus, the circadian rhythm of *nocturnin* gene expression may be mediated by cAMP-dependent phosphorylation of CREB. However, other protein kinases, including Ca^{2+}/calmodulin-dependent kinases and MAP kinases phosphorylate and activate CREB, and a definitive link between cAMP and rhythmic CREB phosphorylation in photoreceptors has yet to be established.

The immediate early gene product, Fos, itself a transcription factor, is regulated in a circadian fashion in photoreceptors. *c-Fos* mRNA levels undergo circadian fluctuations with highest levels at night (Yoshida et al., 1993) and the induction of Fos protein by darkness exhibits circadian dependency (Humphries and Carter, 2004). cAMP induces *c-fos* expression in neurons (Sheng and Greenberg, 1990). Thus, induction of this transcription factor by cAMP may coordinately regulate many photoreceptor genes in a circadian fashion. For example, the circadian rhythm of phospholipid metabolism in chicken photoreceptors appears to be regulated by circadian expression of Fos (Bussolino et al., 1998).

The affinity of cyclic nucleotide-gated channels (CNGCs) for cGMP in cultured chicken cone photoreceptors undergoes a circadian rhythm with highest affinity at night (Ko et al., 2001), and is modulated by dopamine (Ko et al., 2003). cAMP, acting through PKA, Ras, and Erk, is a key player in the circadian regulation of the CNGC (Ko et al., 2004b). However, the nocturnal increase of affinity for cGMP is unaffected by nitrendipine, an L-type Ca^{2+} channel blocker that inhibits the nocturnal increase of AANAT activity, leading to the suggestion that the circadian pathways for the modulation of CNGCs are different from those that control AANAT activity and melatonin synthesis in the

chicken cones (Ko et al., 2004a). One possible explanation for these results is that different pools of cAMP are involved in controlling the two circadian responses. It is unclear if Ca^{2+} is required for the modulation of the CNGC; if so, localized changes in intracellular Ca^{2+} arising from influx through the CNGC or LOT channels may initiate the cAMP cascade that regulates affinity for cGMP.

5. Circadian clock networks in retina

Photoreceptor clocks are not alone in the retina. Emerging evidence indicates the existence of circadian oscillators in many retinal cell types. Some of these oscillators may be autonomous, while others may be secondary to master clocks. For example, lesions of the SCN abolish the circadian expression of retinal *Per2* mRNA, but not that of *AANAT* in the rat retina (Sakamoto et al., 2000). These clocks may interact in networks to regulate circadian retinal physiology. This section will review the evidence for multiple oscillatory mechanisms within the retina and discuss research on a clock network involving photoreceptors and dopamine amacrine cells.

5.1. Clock gene expression in non-photoreceptor cells of retina

Nearly all of the genes identified as components of the core oscillator in the SCN have been identified in retina by Northern blot or RT-PCR (Section 3.2). In a few cases, retinal expression has been localized. In chicken retina, the clock genes *Cry1* and *Cry2* are expressed in inner retinal neurons, as well as in photoreceptors (Bailey et al., 2002; Haque et al., 2002). *Cry1* is rhythmically expressed in RPE, photoreceptors, the ganglion cell layer, and the proximal inner nuclear layer, where amacrine and interplexiform cells are located (Haque et al., 2002). In *Xenopus* retina, *Cry1*, *2*, and *3* are expressed predominantly in photoreceptor cells, but some cells of the inner nuclear and ganglion cell layer express them also (Zhu and Green, 2001). *Xenopus Per1* and *Per2* are widely distributed, with expression in RPE, photoreceptors, inner nuclear layer and ganglion cells (Zhuang et al., 2000). Analysis of *Xenopus* neural retina by in situ hybridization indicates that the antiphasic diurnal expression of *Per1* and *Per2* and light induction of *Per2* occurs primarily in photoreceptor cells, while expression in inner retinal neurons is nearly constitutive (Besharse et al., 2004). Thus, circadian clock gene expression appears to be differentially regulated in different cell types of the retina.

While the existence of circadian clocks in photoreceptor cells is well established in lower vertebrates, it is controversial in mammals. Initial studies of *Per1*, *Clock*, and *Bmal1* mRNA in mouse retina localized expression primarily to inner retina, with lower levels of expression in photoreceptor cells (Gekakis et al., 1998). In rat retina, *Per1* and *Per2* were also found primarily in the inner retina (Namihira et al., 2001). Using a PER1:GFP transgenic mouse, GFP expression, which co-localizes with PER1-like immunoreactivity, was found in the cells of the inner nuclear layer and a few ganglion cells, but not in photoreceptors (Witkovsky et al., 2003). In human and mouse retinas, *Cry1* and *Cry2* are found in ganglion cells and a few cells of the inner nuclear layer (Miyamoto and Sancar, 1998; Thompson et al., 2004). Thus, there is little evidence of clock gene expression in photoreceptor cells of mammals. Nevertheless, mammalian photoreceptors show circadian physiology, including rhythmic outer segment disc shedding (LaVail, 1976), Fos expression (Yoshida et al., 1993; Humphries and Carter, 2004), and melatonin biosynthesis (Tosini and Menaker, 1996; Liu et al., 2004). If mammalian photoreceptors contain autonomous clocks, they may use a different set of clock genes to organize their oscillators. Alternatively, circadian rhythms in photoreceptors may be driven by inner retinal clocks via synaptic or paracrine input. It is of considerable interest that different clock genes appear to be expressed in different cell types, even in the inner retina. Thus, circadian organization in the mammalian retina may involve networks of cells that must interact to form a sustainable oscillator.

5.2. Circadian rhythms in isolated retinal cell types

Isolated photoreceptor cells and ganglion cells of *Xenopus* and chicken contain circadian oscillators. Localization of circadian clock functions to *Xenopus* photoreceptor cells was discussed above. In intact chicken retina, the synthesis of phospholipids in both photoreceptors and ganglion cells is circadian (Guido et al., 2001). The circadian rhythm of phospholipid synthesis was also found in immunopurified cultured ganglion cells, as were rhythms in the activities of several enzymes in the phospholipid synthesis and degradation pathway (Garbarino-Pico et al., 2004a). A subset of chicken ganglion cells also expresses *AANAT* mRNA (Bernard et al., 1997) and immunopurified ganglion cell cultures display a circadian rhythm of *AANAT* transcript (Garbarino-Pico et al., 2004b). Thus, an autonomous circadian clock capable of functioning in isolation appears to exist in chicken ganglion cells. The clockwork machinery used by ganglion cells to generate these oscillations remains to be determined. In addition, it is unclear to what extent this ganglion cell clock is influenced by retinal circuitry or other retinal clocks in vivo.

5.3. Melatonin and dopamine as components of a retinal clock network

Melatonin and dopamine play opposing roles in the regulation of retinal adaptive physiology (Besharse et al., 1988; Cahill and Besharse, 1995; Iuvone, 1995; Green and Besharse, 2004). Dopamine, an amacrine and interplexiform cell neurotransmitter (Ehinger, 1977; Dowling and Ehinger, 1978), functions as a chemical signal for light, producing light-adaptive physiology. Melatonin, on the other hand, has dark-adaptive effects. In many species, the synthesis and release of both melatonin and dopamine are under circadian control, with melatonin released at night and dopamine during the daytime (e.g., Wirz-Justice et al., 1984; Adachi et al., 1998; Ribelayga et al., 2002). Melatonin inhibits the release of dopamine through an action on MT2-like melatonin receptors (Dubocovich, 1983; Boatright et al., 1994; Dubocovich et al., 1997; Ribelayga et al., 2004), and dopamine inhibits the synthesis and release of melatonin from photoreceptor cells by acting on D2/D4-like receptors (Iuvone and Besharse, 1986b; Cahill and Besharse, 1991; Zawilska and Nowak, 1994; Tosini and Dirden, 2000). Dopamine can also induce *Per2* and entrain the photoreceptor circadian clock, showing a phase response curve similar to that of light, in *Xenopus* retina (Cahill and Besharse, 1991, 1993; Steenhard and Besharse, 2000; Besharse et al., 2004), but apparently not in chicken retina (Zawilska, 1994). Thus, the melatonin secreting photoreceptors and dopamine secreting amacrine/interplexiform cells form a cellular feedback loop functioning to regulate circadian retinal physiology (Fig. 7).

The evidence for clock function in photoreceptors in non-mammalian and mammalian vertebrates has been discussed above. An open question is what drives rhythmic dopamine metabolism: an autonomous circadian oscillator in the dopamine neurons or oscillators in other cells, possibly acting through melatonin or synaptic inputs. Dopamine neurons in mouse retina express clock genes. In PER1:GFP mice, rhythmic GFP expression was observed in dopamine amacrine cells in cyclic light and in constant darkness (Witkovsky et al., 2003). Single cell expression analysis also demonstrated the expression of *Cry1* mRNA in dopamine cells and CRY1 protein expression was confirmed by immunocytochemistry (Gustincich et al., 2004). Immunoreactive CRY2, CLOCK, BMAL1, and PER1 were also detected (Gustincich et al., 2004), indicating that most circadian clock genes are expressed in the dopamine neuron. These findings support the hypothesis that dopamine amacrine cells may contain an autonomous circadian clock that drives dopamine metabolism and release. However, retinal dopamine content and metabolism is circadian in C3H mice, which synthesize melatonin, but not in C57Bl/6J or BALB/c mice, which are genetically incapable of synthesizing melatonin (Nir et al., 2000; Doyle et al., 2002a). Dopamine metabolism is diurnal and light-driven in mouse strains with mutations in melatonin-synthesizing enzymes. Moreover, daily injections of melatonin resulted in circadian rhythms of dopamine in retinas of C57Bl/6J mice (Doyle et al., 2002a). These findings suggest that circadian oscillations of dopamine synthesis and metabolism in mouse retina are driven or entrained by rhythmic release of melatonin from photoreceptor cells.

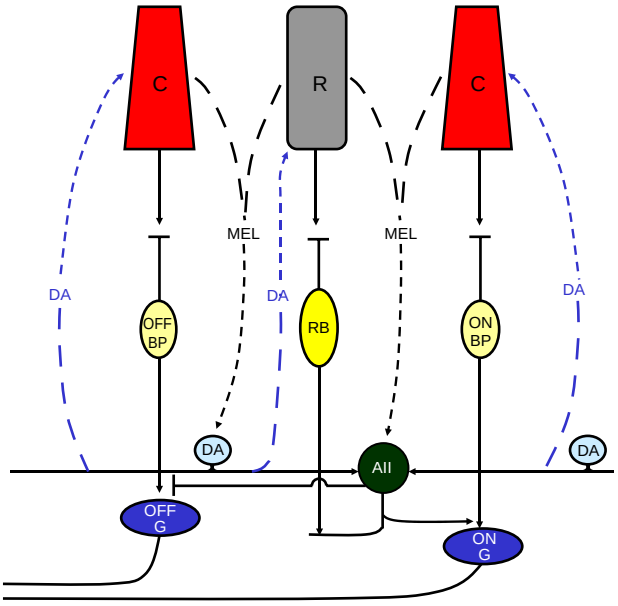

Fig. 7. A melatonin–dopamine circadian clock network. Melatonin (MEL) is released from rod (R) and cone (C) photoreceptor cells at night and diffuses to act on receptors distributed throughout the retina. Among the targets are the dopamine (DA) amacrine and interplexiform cells, where melatonin inhibits DA release (Dubocovich 1983). The DA neurons express MEL receptors (Fujieda et al., 2000), suggesting a direct effect, although input from GABA amacrine cells, which also express MEL receptors, is required for inhibition (Boatright et al., 1994). DA amacrine cells express clock genes and in some species DA release is circadian, with a phase opposite to that of the melatonin rhythm; high release during the daytime (Adachi et al., 1998; Witkovsky et al., 2003). Thus, MEL appears to contribute to the nocturnal suppression of DA release and to its circadian rhythm. The opposite phases of the MEL and DA rhythms appear to coincide with their physiological roles, with DA promoting light adaptive physiology and melatonin dark-adaptive effects. DA, on the other hand, feeds back to the photoreceptor cells to inhibit MEL synthesis, contributing to the suppression of MEL synthesis in the daytime. DA also serves as a zeitgeber that together with light entrains the photoreceptor clock. Thus, this reciprocal paracrine feedback loop may function to stabilize circadian output in the inner and outer plexiform layer. AII amacrine cells express both D1 DA receptors and MT1 MEL receptors (Hampson et al., 1992; Scher et al., 2003). These cells are part of the rod pathway, which functions in dim light and communicates rod signals to ON bipolar (BP) cell terminals and OFF BP terminals to drive ON and OFF ganglion (G) cell responses. MEL and DA probably have opposing effects on AII amacrine cell physiology, as co-expressed D1 DA receptors and MEL receptors in amacrine cells functionally interact in an antagonistic manner (Iuvone and Gan, 1995).

To further explore the role of photoreceptors, circadian dopamine metabolism was examined in RCS rats with hereditary photoreceptor degeneration (Doyle et al., 2002b). Interestingly, robust free-running circadian rhythms of dopamine and its metabolites were observed in both normal and photoreceptor-degenerate retinas. These findings suggest that the oscillators driving circadian dopamine metabolism are not in photoreceptor cells. However, a surprising finding suggests that melatonin may still be involved in generating the dopamine rhythms in the RCS rat retina. In normal rat retina, *AANAT* mRNA is expressed primarily in photoreceptors (Niki et al., 1998). The transcript is expressed rhythmically in the photoreceptor layer, but constitutively at a much lower level in the inner nuclear layer (Liu et al., 2004). Following photoreceptor degeneration in the RCS rat, *AANAT* mRNA expression in the inner nuclear layer is up-regulated and becomes rhythmic (Sakamoto et al., 2004a). Circadian rhythms of *AANAT* level and melatonin release persist in degenerated RCS retinas, but the peak of the rhythms is advanced approximately 4 h relative to that in intact retina. Thus, the circadian network of the retina reorganizes following photoreceptor degeneration. The dopamine rhythms may be driven by melatonin synthesized rhythmically in photoreceptor cells in the intact retina and by melatonin synthesized rhythmically by inner nuclear layer cells following photoreceptor degeneration.

Similar conclusions can be drawn from studies on lower vertebrates. In *Xenopus* retina, the reduction of dopamine release at night in darkness is reduced by luzindole, a melatonin receptor antagonist (Boatright et al., 1994). Goldfish, pigeon, and Japanese quail retinas exhibit robust circadian rhythms of endogenous dopamine release with highest levels during the daytime (Adachi et al., 1995, 1998; Ebihara et al., 1997; Ribelayga et al., 2002). The rhythm of dopamine release mediates the circadian rhythm of rod–cone dominance in the retina (Witkovsky et al., 1988; Manglapus et al., 1999; Ribelayga et al., 2002). In goldfish, the circadian rhythm of dopamine release is blocked by both melatonin and luzindole (Ribelayga et al., 2004). In the continuous presence of exogenous melatonin, dopamine release is low and arrhythmic, with levels at all times of day similar to those at nighttime in controls. In the continuous presence of the melatonin receptor antagonist luzindole, dopamine release is high and arrhythmic, with levels comparable to daytime release in controls.

According to the above, circadian melatonin receptor occupation appears necessary for generating the rhythm of dopamine release. However, in pigeon, the intraocular injection of quinpirole, a D2-like dopamine receptor agonist, reduces melatonin secretion at all times of the night, but differentially affects dopamine release in a time-dependent manner (Adachi et al., 1999). Quinpirole increases dopamine when injected at the beginning (ZT 12) and near the end (ZT 21) of the night, but not in the middle of the night (ZT 15 or 18). Thus, inhibiting melatonin release and decreasing melatonin receptor occupancy in the middle of the night is not sufficient to stimulate dopamine release, leading to the suggestion that dopamine release may be controlled by its own oscillators, separate from those regulating melatonin (Adachi et al., 1999). The results in goldfish and pigeon are not necessarily contradictory. A possible explanation is that circadian oscillations of clock genes in dopamine amacrine cells may be entrained or driven by melatonin receptor occupancy. There is precedence for this type of control in other tissues. For example, melatonin acts in concert with adenosine to generate rhythms of clock gene expression in the *pars tuberalis* of the mouse (Stehle et al., 2002). Melatonin receptor occupancy can shift the phase the clock in the rat SCN (McArthur et al., 1991; Liu et al., 1997; Hunt et al., 2001). Also, the rat pineal gland shows rhythmic expression of clock genes and melatonin synthesis in response to circadian occupancy of adrenergic receptors (Klein et al., 1970; Deguchi and Axelrod, 1972; Fukuhara et al., 2000, 2002; Simonneaux et al., 2004). The role of melatonin in clock gene expression in the dopamine amacrine cell is yet to be explored.

6. Future directions

Much remains to be learned about circadian organization of the retina. Questions remain unanswered about entrainment mechanisms, clockwork machinery, and output mechanisms. The photopigments and signaling mechanisms involved in retinal circadian clock entrainment have yet to be conclusively identified. In rods and cones, this might be mediated by traditional opsins. Alternatively, novel photopigments might regulate entrainment of photoreceptor clocks. Clocks in inner retinal neurons might be entrained by multisynaptic input from rods and cones, by paracrine factors such as melatonin, or by novel photopigments expressed in inner retinal neurons. The widespread distribution of melanopsin and the cryptochromes, CRY1 and CRY2, in chicken retina (Haque et al., 2002; Chaurasia et al., 2005) make these attractive candidates. Crytochromes entrain circadian clocks in *Drosophila* (Emery et al., 1998; Stanewsky et al., 1998) and melanopsin-containing photosensitive ganglion cells have been implicated in the entrainment of the clock in the SCN of mammals (Berson et al., 2002; Hattar et al., 2003; Panda et al., 2003). Melanopsin mRNA expression is regulated by circadian clocks in retinas of chicken and rat (Sakamoto et al., 2004b; Chaurasia et al., 2005), suggesting an association with retinal circadian function. Elucidating

the signaling pathway(s) that mediate entrainment of retinal circadian clocks represents an important area of future research. In addition, determining if mammalian photoreceptors contain circadian clocks is an important goal, and if they do, identifying the molecular clockwork that generates the rhythms is likely to further our understanding of circadian retinal organization.

We are beginning to understand how melatonin synthesis is regulated in retina, but many details still remain unknown. The functions of melatonin and *N*-acetylserotonin in the retina are also poorly understood, in part due to the scarcity of specific pharmacological tools to probe their actions. It seems likely that many more circadian outputs will be identified and that other neuromodulators are regulated by circadian clocks. One such candidate is adenosine. In *Xenopus* retina, adenosine release appears to be circadian, with high levels at night contributing to the stimulation of melatonin biosynthesis (Iuvone et al., 2000). Adenosine release has also been shown to be circadian in fish retina where, together with dopamine, it modulates the rhythm of rod–cone inputs to horizontal cells (Mangel et al., 2002; Ribelayga and Mangel, 2004).

In retinas that do not make melatonin, it will be important to determine whether or not the rhythmic production of *N*-acetylserotonin substitutes for melatonin, and how the detoxification influence of AANAT impacts retinal physiology and photodetection. Lastly, it will be important to determine if and how multiple retinal clocks interact to regulate circadian retinal physiology and to examine the impact of circadian function and dysfunction on retinal pathology.

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